STUDIES OF PHOTODYNAMIC ACTION

II. The Relationship between Hemolysis by Irradiated and Non-Irradiated Eosine

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Many photodynamic substances when in sufficient concentration bring about changes in cells which are similar to the changes produced by the irradiated dye in less concentration. This effect was studied very early by Tappeiner and Jodlbauer (1904), Jodlbauer and Busck (1905) and others. Sacharoff and Sachs described hemolysis by non-irradiated erythrosine and other photodynamic substances in the paper in which they first described photodynamic hemolysis (1905). Jodlbauer and Haffner (1921*a*) summarize a certain number of such studies and point out that among photodynamic substances, those which bring about hemolysis in the lowest concentration when not irradiated are generally the most active in bringing about photodynamic phenomena in living cells. Likewise, they are most active in flocculating the colloids of hemolyzed blood cells. While this generalization has many exceptions, it indicates a correlation between the action of irradiated and non-irradiated photodynamic substances.

Jodlbauer and Haffner (1921*b*) studied the effect of hydrogen ion concentration on hemolysis by non-irradiated eosine and rose bengale (tetra-brom tetra-iodo fluorescein). They found that the hemolytic effect, as measured by the minimum dye concentration necessary to produce hemolysis, was at a minimum in the region of neutrality and increased as the reaction became either more acid or more alkaline. This increase was more pronounced on the acid side. In acid solutions of dye in high concentrations, fixation was found to take place instead of hemolysis. The fixing action, like the hemolytic action, increased with increasing acidity. These investigators did not examine the effect of hydrogen ion concentration or dye concentration on the hemolytic and fixing activity of the irradiated dyes. This has been done in the experiments described below, the results of which point to another correlation between the action of irradiated and non-irradiated dyes, namely, that the hemolytic and fixing action of the irradiated dye is modified in the same way by hydrogen ion concentration as is that of the non-irradiated dye.

Experimental

Effect of Hydrogen Ion Concentration on Hemolysis and Fixation by Irradiated Eosine.—In a preceding paper (Blum, 1930), it was shown that previously irradiated eosine may bring about hemolysis to a degree less than that occurring when the dye is irradiated together with the

Table I

Effect of Hydrogen Ion Concentration on Hemolytic Action of Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 7 hours in dark following irradiation. *H* = complete hemolysis; (*H*) = partial hemolysis; *a* = cells plus eosine irradiated; *b* = eosine irradiated alone, cells added immediately after exposure; *c* = non-irradiated. *a* and *b* irradiated 1 hour (11:00 A.M.–12:00 M., Sept. 15, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.4			pH 6.9			pH 7.3			pH 7.6		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1.0 (0.0014 M)	—	—	—	—	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	(*H*)
0.5 (0.0007 M)	—	—	—	(*H*)	*H*	(*H*)	(*H*)	*H*	(*H*)	(*H*)	*H*	—	(*H*)	(*H*)	—
0.25 (0.00035 M)	(*H*)	—	—	*H*	*H*	(*H*)	*H*	*H*	—	(*H*)	*H*	—	(*H*)	(*H*)	—
0.125 (0.00017 M)	(*H*)	*H*	—	*H*	*H*	(*H*)	*H*	*H*	—	(*H*)	(*H*)	—	(*H*)	*H*	—
0.062 (0.00009 M)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.031 (0.00004 M)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.015 (0.00002 M)	*H*	(*H*)	—	*H*	*H*	—	*H*	(*H*)	—	*H*	(*H*)	—	*H*	(*H*)	—
0.007 (0.00001 M)	*H*	(*H*)	—	*H*	(*H*)	—	*H*	—	—	*H*	(*H*)	—	*H*	(*H*)	—
0.004 (0.000005 M)	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.002 (0.000002 M)	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

cells. Therefore, parallel series of experiments were conducted under both conditions together with a like series, using the non-irradiated dye. The technique of the experiments was the same as that described in the above paper. A much narrower range of hydrogen ion concentration was studied than that examined by Jodlbauer and Haffner (1921*b*), as it seemed wise to avoid as much as possible the hemolytic effects of the hydrogen and hydroxyl ions themselves. Within the range here used, pH 6.0 to pH 7.7, no great difference in cell volumes occurs in

the solutions used.[1] Thus we may assume that the hemolytic action of hydrogen and hydroxyl ions is practically negligible in our experiments.

Three like series of dilutions of the dye were used at each hydrogen ion concentration; (*a*) dye irradiated together with cells, (*b*) dye irradiated alone, cells added subsequently in the dark and (*c*) dye not

TABLE II

Effect of Hydrogen Ion Concentration on Hemolytic Action of Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 6 hours in dark following irradiation. *P* = precipitate following observation of complete hemolysis at an earlier time. Other symbols as in Table I. *a* and *b* irradiated 1 hour and 15 minutes (1:45–3:00 P.M., May 10, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.5			pH 7.0			pH 7.4			pH 7.7		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
4.0	—	—	—				—	*P*	*P*				*H*	*H*	*H*
2.0				—	—	—				*H*	*H*	*H*			
1.0	—	—	—				—	(*H*)	*H*				(*H*)	*H*	(*H*)
0.5				—	*H*	*H*				(*H*)	(*H*)	—			
0.25	—	—	—				(*H*)	—	—				—	(*H*)	—
0.125				(*H*)	(*H*)	—				(*H*)	—	—			
0.062	—	—	—				(*H*)	—	—				(*H*)	—	—
0.031				(*H*)	—	—				(*H*)	—	—			
0.015	—	—	—				(*H*)	—	—				(*H*)	—	—
0.007				*H*	—	—				(*H*)	—	—			

irradiated. Tables I and II are taken from typical experiments covering overlapping regions of eosine concentration, and do not agree absolutely in a quantitative sense as regards the irradiated dye. The conditions of irradiation never being the same in any two experiments, absolute agreement is never obtained, but each experiment performed demonstrates certain qualitative differences between the irradiated and non-irradiated dye. A certain parallelism may be observed in the action of the irradiated and non-irradiated dye in that, generally speaking, hemolysis occurs most readily in both cases at the same hydrogen ion concentrations; the irradiated solutions, however, show hemolysis at lower dye concentrations. The solutions irradiated alone (*b*) always appear to take a place intermediate between the non-irradiated dye (*c*) and the dye irradiated with the cells (*a*) as regards their hemolytic activity. The explanation of this latter fact has been discussed by the writer (1930).

[1] See Blum (1930).

We note in both Tables I and II, at acid reactions, that hemolysis occurs in higher concentrations in non-irradiated than in irradiated solutions. Here again series *b* appears to be intermediate between *a* and *c*. This apparently paradoxical behavior is due to fixation of the cells, as described by Jodlbauer and Haffner (1921*b*) for high concentrations of non-irradiated eosine and rose bengale at acid reactions. This is demonstrated in the following type of experiment which was carried out in the usual manner, except that after several hours in the dark following irradiation the cells were examined for fixation by treatment with distilled water. The solutions were pipetted off from all those tubes in which complete hemolysis had not occurred, leaving only the cells and debris in the bottom of the tubes. The solutions were then replaced by distilled water and allowed to stand for several hours. At the end of this time, the tubes were again examined for hemolysis, the determinations with the naked eye being checked by microscopic examination. The cells in those tubes which did not show hemolysis before or after this treatment were considered as completely fixed and are designated in Table III by the letter *F*. Those tubes which showed some hemolysis, but in which intact cells could be observed, are represented by (*F*) to indicate partial fixation of the cells. Partial fixation was also considered to have occurred in those tubes which showed hemolysis before treatment with distilled water but no hemolysis after, and these are likewise designated by (*F*). Partial hemolysis before this treatment is designated by (*H*), and complete hemolysis by *H* as in the previous tables.

This treatment of the cells is rather severe and selection of a criterion of fixation is rather arbitrary. Nevertheless, the results demonstrate in a striking manner that fixation, like hemolysis, is shifted into a lower concentration of the dye by irradiation. They also show that fixation is most apparent in both irradiated and non-irradiated dye at the same hydrogen ion concentration, and that series *b* is again intermediate between *a* and *c* as in the case of hemolysis. A consideration of the data in Table III indicates the fallacy of adopting hemolysis as a quantitative measure of photodynamic action. Very false deductions may be based upon the simple observation that hemolysis does not occur under a given set of conditions, since this may indicate either that the action of the photodynamic substance is too weak to produce hemolysis, or that it has produced fixation instead. We are obviously dealing with two manifestations of the action of eosine on red blood cells which are both modified in much the same way by irradiation and by hydrogen ion concentration.

Still a third manifestation of the action of non-irradiated and ir-

radiated dyes appears in the precipitation of cell constituents from suspensions of hemolyzed blood cells. Jodlbauer and Haffner (1921*b*) have described flocculation of hemolyzed cells by non-irradiated eosine and rose bengale. Table IV shows the results of an experiment which affords a comparison between the action of irradiated and non-irradiated eosine on hemolyzed cells. This experiment was conducted simul-

TABLE III

Fixation and Hemolysis of Red Blood Cells by Irradiated and Non-Irradiated Eosine.

All solutions contain sodium phosphate buffer, isosmotic with 0.15 M NaCl. Observations made after 7 hours and 30 minutes in dark following irradiation. *F* = complete fixation; (*F*) = partial fixation. Other symbols as in Tables I and II. *a* and *b* irradiated 2 hours and 15 minutes (October 11, 1929).

Concentration of Eosine *per cent*	pH 6.0			pH 6.5		
	a	*b*	*c*	*a*	*b*	*c*
2.0	*F*	*F*	*F*	(*F*)	(*H*) (*F*)	(*F*)
1.33	*F*	*F*	*F*	(*H*) (*F*)	(*H*)	(*H*)
0.89	*F*	*F*	*F*	(*H*) (*F*)	*H*	*H*
0.59	*F*	*F*	(*F*)	(*H*) (*F*)	*H*	(*H*)
0.39	*F*	(*H*) (*F*)	(*F*)	(*H*)	*H*	(*H*)
0.26	*F*	*H*	—	(*H*)	*H*	—
0.17	*F*	*H*	—	(*H*)	*H*	—
0.11	(*H*)	*H*	—	(*H*)	*H*	—
0.075	(*H*)	*H*	—	(*H*)	*H*	—
0.050	(*H*)	*H*	—	(*H*)	*H*	—
0.	—	—	—	—	—	—

taneously with the one described in Table II and under exactly the same conditions, except that blood cells hemolyzed with distilled water were used instead of intact blood cells. It may be readily seen that precipitation, like hemolysis and fixation, is more pronounced in irradiated than in non-irradiated solutions of the dye, and that series *b* is intermediate between *a* and *c*. Again, as with hemolysis and fixation, precipitation is most pronounced at the same hydrogen ion concentrations in both the irradiated and the non-irradiated dye.

Table V is a record from the experiment described in Table I but represents observations made five hours later or after twelve hours in the dark following irradiation. This table is inserted to show how precipitation may mask the occurrence of hemolysis, and to emphasize the importance of making more observations than one in the course of a single experiment. Tubes often show partial hemolysis at one period with subsequent precipitation of the hemolyzed cell constituents which may mask the presence of unhemolyzed fixed cells.

Table IV

Effect of Hydrogen Ion Concentration on Precipitation of Hemolyzed Cell Constituents by Irradiated and Non-Irradiated Eosine.

Symbols as in preceding tables. *P* = precipitate. *a* and *b* irradiated 1 hour and 15 minutes (1:45–3:00 P.M., May 10, 1929). All solutions contain sodium phosphate buffer isosmotic with 0.15 M NaCl. Observations made after 6 hours in dark following exposure of *a* and *b*.

Concentration of Eosine *per cent*	pH 6.0			pH 6.5			pH 7.0			pH 7.4			pH 7.7		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
4.0	*P*	*P*	*P*				*P*	*P*	*P*				—	—	—
2.0				*P*	*P*	—				slight *P*	—	—			
1.0	*P*	*P*	*P*				*P*	*P*	—				—	—	—
0.5				*P*	*P*	—				—	—	—			
0.25	*P*	*P*	slight *P*				*P*	*P*	—				—	—	—
0.125				—	—	—				—	—	—			
0.062	*P*	trace *P*	—				—	—	—				—	—	—
0.031				—	—	—				—	—	—			
0.015	*P*	—	—				—	—	—				—	—	—
0.007				*P*	—	—				—	—	—			

Table V

Effect of Hydrogen Ion Concentration on Hemolytic Activity of Irradiated and Non-Irradiated Eosine.

Same experiment described in Table I. Observations made after 12 hours in dark following irradiation. Symbols as in preceding tables.

Concentration of Eosine *per cent*	pH 6.0			pH 6.4			pH 6.9			pH 7.3			pH 7.6		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1.0	*P*	*P*	—	*P*	*H*	*P*	*P*	*H*	*H*	*H*	*H*	*H*	*H*	*H*	(*H*)
0.5	*P*	*P*	—	*P*	*H*	(*H*)	*P*	*H*	(*H*)	*H*	*H*	(*H*)	*H*	*H*	(*H*)
0.25	*P*	*P*	—	*H*	*H*	(*H*)	*P*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.125	*P*	*P*	—	*H*	*H*	(*H*)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.062	*P*	*P*	—	*H*	*H*	(*H*)	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—
0.031	*P*	*P*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	*H*	—
0.015	*H*	*H*	—	*H*	*H*	—	*H*	*H*	—	*H*	(*H*)	—	*H*	(*H*)	—
0.007	*H*	(*H*)	—	*H*	(*H*)	—	*H*	—	—	*H*	(*H*)	—	*H*	(*H*)	—
0.004	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.002	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—	*H*	—	—
0.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Changes in Hydrogen Ion Concentration of Eosine Solutions during Irradiation.—Since hydrogen ion concentration exerts a marked effect on the hemolytic action of both irradiated and non-irradiated eosine, changes in hydrogen ion concentration during irradiation of unbuffered solutions might be expected to markedly affect the hemolytic activity of such solutions. Such changes in hydrogen ion concentration during the course of irradiation may be readily demonstrated in dilute unbuffered solutions of eosine by means of indicators. In 0.0005 M eosine solution, the sulphonthalein indicators may be used with sufficient accuracy to observe differences of hydrogen ion concentration corresponding to 0.5 pH. The procedure in such determinations was to add the indicators to samples of irradiated and non-irradiated dye, and to compare these to buffered solutions containing the same concentration of dye and indicator. The shift of hydrogen ion concentration during irradiation was always found to be toward the acid side. In one set of experiments, unbuffered 0.0005 M eosine solutions which had a hydrogen ion concentration of approximately pH 6.5 before irradiation were found to have increased in hydrogen ion concentration to approximately pH 4.0 in the course of five hours' irradiation. The latter value is far outside the range of hydrogen ion concentrations examined in the above experiments.

Since most of the experiments of previous investigators of photodynamic processes have been carried out in unbuffered solutions, it is quite probable that such change in hydrogen ion concentration may have been a very important factor in the results obtained. It is probable that some of the failures of other investigators to produce hemolysis by previously irradiated photodynamic substances, as described by the writer (1930), may have been due to the shifting of the hydrogen ion concentration into an acid region in which fixation and not hemolysis occurs with the concentration of the dye used. The writer has, in fact, observed fixation of cells added to previously irradiated unbuffered solutions of eosine. This criticism applies to the experiments of Sacharoff and Sachs (1905), Hausmann (1909 and 1910) and Hasselbach (1909), who were unable to demonstrate hemolysis with previously irradiated solutions of numerous photodynamic substances.

Hemolysis and Fixation by Hydrogen Peroxide and Non-irradiated Eosine.—In the preceding paper of this series (Blum 1930), the probability was discussed that the alteration in hemolytic activity of eosine by irradiation is due to an increase in oxidizing power of the dye solution, resulting in the oxidation of cell constituents. From the experiments described above it appears that the effect of irradiation is simply to increase the effects produced by non-irradiated eosine, evi-

denced by the shifting of hemolysis, fixation, and precipitation into lower concentrations of the dye. This suggests that the action of the non-irradiated eosine is also an oxidation which is increased by irradiation. This is probably not the case, however, since the non-irradiated dye is not capable of oxidizing a readily oxidizable substance such as the iodide ion. Furthermore, at least certain of the changes produced by the non-irradiated dyes may be readily reversed by simply washing out the dye, (Kudo and Jodlbauer, 1908). A possible explanation of the similarity of action between irradiated and non-irradiated eosine is that irradiation brings about oxidative changes which are simply superimposed upon changes brought about by the non-irradiated dye. This assumption need not take into consideration the mechanism of the changes brought about by the non-irradiated dye.

Such an assumption can be tested to a certain extent by finding whether oxidation alone may bring about hemolysis, and whether the effect of this oxidation is increased by the presence of non-irradiated eosine. Hydrogen peroxide is capable of oxidizing iodide ion and must, therefore, have a greater oxidizing power than non-irradiated eosine. The following experiment shows the result of treating red blood cells with hydrogen peroxide and with hydrogen peroxide plus non-irradiated eosine. Series of dilutions of hydrogen peroxide and of eosine were used. The hydrogen peroxide solutions were freshly prepared from " Superoxol " (Merck) containing 30 per cent hydrogen peroxide, which was neutralized with 0.1 N sodium hydroxide and diluted with the usual mixture of primary and secondary sodium phosphates. The greatest concentration of hydrogen peroxide used in the experiments was 3 per cent, which represents a dilution of salt content of approximately 10 per cent. Much greater dilutions with water are necessary to bring about hemolysis, and as a matter of fact fixation and not hemolysis occurs at this concentration of hydrogen peroxide. In the lower dilutions of hydrogen peroxide which were used, the reduction of salt content is certainly negligible. The technique of preparation and addition of blood cells was the same as described for previous experiments. Table VI shows the result of an experiment in which the hydrogen ion concentration of the solutions was buffered at pH 6.0. It will be noted that with the peroxide solutions containing no eosine, hemolysis occurs at concentrations of 0.37 per cent to 1.5 per cent inclusive, while above this at 3 per cent fixation occurs. At the latter concentration the hemoglobin is changed to a brownish substance, probably methemoglobin. This confirms the findings of both Schmidt and Norman (1922) and Rigoni (1926). The former found that no hemolysis took place in cells treated with hydrogen peroxide (concen-

Table VI

Hemolysis and Fixation of Cells by Hydrogen Peroxide and Non-Irradiated Eosine

Symbols as in Table III. All solutions contain sodium phosphate buffer pH 6.0, isosmotic with 0.15 M NaCl. Observations made six hours after addition of blood cells to eosine- peroxide mixtures.

Eosine	H_2O_2										
	3.0% (0.9 M)	1.5% (0.45 M)	0.75% (0.22 M)	0.37% (0.11 M)	0.19% (0.05 M)	0.09% (0.03 M)	0.05% (0.015 M)	0.02% (0.007 M)	0.01% (0.004 M)	0.005% (0.002 M)	0.%
per cent											
1.0 (0.0014 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
0.5 (0.0007 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
0.25 (0.00035 M)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	(*F*)	—	—	—
0.125 (0.00017 M)	*F*	*F*	*F*	*F*	*F*	(*II*) (*F*)	—	—	—	—	—
0.062 (0.00009 M)	*F*	*F*	*F*	(*II*) (*F*)	(*II*) (*F*)	—	—	—	—	—	—
0.031 (0.00004 M)	*F*	*F*	(*II*) (*F*)	*II*	*II*	(*II*)	(*II*)	—	—	—	—
0.015 (0.00002 M)	*F*	(*II*) (*F*)	*II*	*II*	*II*	*II*	(*II*)	—	—	—	—
0.007 (0.00001 M)	*F*	(*II*)	(*II*)	*II*	*II*	*II*	*II*	(*II*)	(*II*)	(*II*)	—
0.004 (0.000005 M)	*F*	*II*	*II*	*II*	*II*	*II*	*II*	*II*	—	(*II*)	—
0.002 (0.000002 M)	*F*	*II*	*II*	*II*	*II*	*II*	*II*	*II*	—	—	—
0.	*F*	*II*	*II*	(*II*)	—	—	—	—	—	—	—

tration not stated) but that methemoglobin formation occurred. The latter found hemolysis in only a given range of hydrogen ion concentrations, stronger as well as weaker solutions failing to bring it about. We see, thus, that an oxidizing agent alone may produce both hemolysis and fixation.

Further examination of Table VI shows that the addition of non-irradiated eosine to the peroxide greatly alters the region of the concentration at which hemolysis and fixation occur. The presence of the dye shifts the region of hemolysis and that of fixation into much lower concentrations of peroxide. Likewise the presence of peroxide shifts these phenomena into lower eosine concentration. Thus, peroxide plus eosine may accomplish hemolysis or fixation in concentrations at which neither alone is effective.

Treatment with hydrogen peroxide plus non-irradiated eosine markedly alters the solubility of hemolyzed blood cell constituents. Decrease of solubility is evidenced by precipitates varying from slight cloudiness to heavy flocculation. While the results at hand demonstrate a definite decrease in solubility at certain concentrations of hydrogen peroxide plus eosine as compared to the same concentration of eosine alone, a more careful analysis does not seem justifiable without further data.

From these experiments it seems clear that the hemolyzing and fixing effect of non-irradiated eosine may be increased by the action of an oxidizing agent such as hydrogen peroxide. We see also that oxidation alone may produce hemolysis and fixation. This evidence seems to support our assumption that the oxidations brought about by the irradiation of the dye are merely superimposed upon the action of the non-irradiated dye. Evidence was offered by the writer (1930) that in the presence of potassium iodide, irradiated eosine returns to its non-irradiated form after having oxidized a corresponding quantity of iodide ion. Similarly, we must assume that if an oxidation of cell constituents takes place, the irradiated dye must return to its non-irradiated form after having oxidized these substances. Thus we may consider the dye as playing a dual rôle, first as the non-irradiated dye, and secondly as an oxidizing agent produced by irradiation.

As shown in the above paper, the oxidizing power of previously irradiated eosine is never greater than the molecular equivalency of the dye. Consequently, we should expect that if the irradiated eosine has an oxidizing power of the same order as hydrogen peroxide, the two substances should bring about hemolysis in approximately the same concentration if the effect of the non-irradiated eosine is neglected. On the basis of the above hypothesis the hemolytic effect of the pre-

viously irradiated eosine is a summation of the oxidation and the effect of the non-irradiated dye, and hence, we should compare the irradiated eosine to peroxide plus an equal concentration of the non-irradiated dye. Comparison of Tables I and VI shows, however, that the concentrations of hydrogen peroxide required to produce hemolysis are many times greater than the concentrations of previously irradiated eosine which produce hemolysis under comparable conditions. Table I shows that at pH 6.0, previously irradiated eosine brings about hemolysis in concentrations as low as 0.00001 M. On the other hand, Table VI shows that, at the same hydrogen ion concentration, 0.002 M hydrogen peroxide is required to bring about partial hemolysis in the presence of 0.00001 M non-irradiated eosine. At slightly lower peroxide concentrations, hemolysis no longer occurs in any eosine concentration which alone does not produce hemolysis. It would thus appear that irradiated eosine is actually a much more powerful oxidizing agent than hydrogen peroxide. Noack (1920) has found, similarly, that irradiated eosine was more effective in oxidizing the sap of *Aloe soccotrina* than was hydrogen peroxide.

Discussion

The correlation made by Jodlbauer and Haffner (1921*a*) between the photodynamic activity of substances and their ability to hemolyze red blood cells in the dark, is readily explained on the basis of the hypothesis outlined above. Since the total observable effect produced by irradiated dyes would be a summation of the effect of the non-irradiated dye and the oxidative changes brought about concomitant with irradiation, an increase in the first of these factors would result in an increase of the total effect. Thus, other factors being equal, the substances having the greatest hemolytic activity in the non-irradiated state should also show the greatest hemolytic effect when irradiated.

Jodlbauer (1926) suggests a quite different explanation of this correlation. He assumes that photodynamic action is dependent upon adsorption, on the basis of the work of Jodlbauer and Haffner (1921*b*), which indicated that the hemolytic action of non-irradiated eosine and rose bengale is dependent upon an adsorption process. He suggests (1) that the dye must be adsorbed by the cells, and (2) must retain its ability to be activated by light while in combination with the cell, thus bringing about oxidative changes. Only those dyes capable of both are photodynamically active. Those dyes which are adsorbed most readily are the most active in bringing about hemolysis in the dark, but all of these do not meet the second qualification. Thus a large number of exceptions to the general rule are accounted for. However,

the hemolysis of red blood cells by previously irradiated fluorescein dyes, described by the writer (1930), renders Jodlbauer's assumption untenable, since contact of the cells and dye during irradiation is not requisite in order to produce hemolysis. The intimate contact between cells and photodynamic substance may assist in the reaction when cells and dye are irradiated together, but offers no explanation for hemolysis when the dye is separately irradiated. The hypothesis presented above offers a more satisfactory explanation of the correlation between the action of the irradiated and non-irradiated dye, in consideration of these facts.

The fact that non-irradiated photodynamic substances may produce definite changes in living cells, has been apparently disregarded by a large number of the investigators of photodynamic action. It seems, however, very important to keep this fact constantly in mind in the interpretation of photodynamic processes. This is particularly true, if we consider the effects of irradiation to be superimposed upon the effects of the non-irradiated dye as here suggested. Whatever the nature of the action of the non-irradiated eosine in producing hemolysis and fixation, we are certain that it may precipitate cell constituents, as shown by the formation of precipitates with hemolyzed cells. The fact that hemolysis and fixation are shifted in the same way by hydrogen ion concentration and by irradiation as is precipitation, suggests that the three processes are closely connected, and the possibility that the two former may be dependent upon changes in the solubility of cell constituents. At any rate, such alterations in solubility of cell constituents must be considered as a possible result of the treatment of biological substances with eosine and other photodynamic substances. We must also consider the possibility of oxidation and of changes in hydrogen ion concentration when biological substances are subjected to the action of irradiated solutions of these photodynamic agents. It is hardly possible to imagine three factors better calculated to alter cell processes than these. It seems, therefore, that until much more careful studies of the varied photodynamic phenomena are made, we need not seek farther for their explanation. It is at least unnecessary to assume unknown processes until the possibilities of the three above-mentioned clearly demonstrable factors are exhausted.

Summary

1. Hemolysis is not the only manifestation of the photodynamic effect of eosine on red blood cells, fixation and precipitation of the

hemolyzed cell constituents also occurring at proper concentrations of the dye.

2. Hemolysis, fixation, and precipitation of the cell constituents are brought about by non-irradiated eosine at proper hydrogen ion concentration and concentration of the dye. Irradiation shifts the region of occurrence of these phenomena into lower concentrations of the dye.

3. In unbuffered solutions of eosine the hydrogen ion concentration increased in the course of irradiation.

4. Hydrogen peroxide may produce hemolysis and fixation in proper concentrations.

5. Hydrogen peroxide and eosine may reënforce each other in producing hemolysis and fixation.

6. Photodynamic action may probably be regarded as a summation of the effects of the non-irradiated photodynamic substance together with the oxidative changes brought about by this substance due to irradiation.

7. Oxidative changes in cell constituents, and changes in hydrogen ion concentration, together with the change in solubility of cell constituents brought about by the photodynamic substance, may possibly account for all the phenomena generally classed under the terms *photodynamic action* or *photodynamic sensitization.*

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